Comprehensive Measurement of Microbial Burden in Nutrient-Deprived Cleanrooms

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Spacecraft surfaces that are destined to land on potential life-harboring celestial bodies are required to be rigorously cleaned and continuously monitored for spore bioburden as a proxy for spacecraft cleanliness. The NASA standard spore assay (NSA), used for spacecraft bioburden estimates, specifically measures spores that are cultivable, aerobic, resistant to heat shock, and grow at 30˚C in a nutrient-rich medium. Since the vast majority of microorganisms cannot be cultivated using the NSA assay, it is necessary to utilize state-of-the art molecular techniques to better understand the presence of all viable microorganisms, not just those measured with the NSA. In this study, the nutrient-deprived low biomass cleanrooms, where spacecraft are assembled, were used as a surrogate to spacecraft surfaces to measure the ratio of NSA spores in relation to the total viable microorganism population to compare with a 2006 space studies report that estimates that for every 1 spore there is approximately 50,000 viable organisms. Ninety-eight surface wipe samples were collected from the spacecraft assembly facility (SAF) cleanroom at the Jet Propulsion Laboratory (JPL) over a 6-month period. The samples were processed and analyzed using classical microbiology along with molecular assays. Traditional microbiology plating methods were used to determine the cultivable bacterial, fungal, and spore populations. Molecular assays were used to determine the total organisms (TO, dead and live) and the viable organisms (VO, live). The TO was measured using adenine triphosphate (ATP) and quantitative polymerase chain reaction (qPCR) assays. The VO was measured using internal ATP, propidium monoazide (PMA)-qPCR, and flow cytometry (after staining for viable microorganisms) assays. Based on the results, it was possible to establish a ratio between spore counts and VO for each viability assay. The ATP based spore to VO ratio ranged from 149 – 746 and the bacterial PMA-qPCR assay based ratio ranged from 314 – 1491 VO. The most conservative estimate came from FACS, which estimated the ratio to be 12,091 VO per 1 NSA spore. Since archaeal (<1%) and fungal (~2%) populations were negligible, the spore to VO ratios were based on bacterial population estimates. The most conservative ratio from this study can be used as a replacement for the SSB estimate on nutrient-deprived (oligotrophic) desiccated spacecraft surfaces, to estimate the VO from NSA measurements without utilizing state-of-the art molecular methods that are costly and require more biomass than is typically found of spacecraft surfaces.

Nomenclature

ATLO	=	Assembly, Test, and Launch Operations
ATP	=	Adenine Triphosphate
BDL	=	Below Detection Limit
CFU	=	Colony Forming Units
DNA	=	Deoxyribose Nucleic Acid
FACS	=	Fluorescent Assisted Cell Sorting

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glyTE = Glycerol Tris EDTA
HFPS = Hollow Fiber Polysulfone
ITS = Internal Transcribed Spacer
JPL = Jet Propulsion Laboratory
LoCoS = Low Coverage Sequencing
MSL = Mars Science Laboratory
NHS = Non-Heat Shock
NSA = NASA Standard Spore Assay
PDA = Potato Dextrose Agar
PMA = Propidium Monoazide
QC = Quality Control
qPCR = Quantitative Polymerase Chain Reaction
RLU = Relative Luminescent Unit
rRNA = Ribosomal Ribonucleic Acid
RSG = RedoxSensor Green
SAF = Spacecraft Assembly Facility
SAG = Single Amplified Genome
TO = Total Organisms
TSA = Tryptic Soy Agar
VO = Viable Organisms
WGA = Whole Genome Amplification

I. Introduction

Since the beginning of planetary protection efforts in the late 1960s, spacecraft biological cleanliness has been measured using traditional microbiology techniques[1-12]. The NASA standard spore assay (NSA), a colony count method, involves collecting spacecraft samples, heat shocking at 80°C for 15 minutes, plating on nutrient rich agar, and growing for 72 hours at 30˚C[13]. Standard cultivation techniques detect only ~1% of the total microbial population that are present in an environment and fail to isolate fastidious microorganisms that may require specific cultivation conditions, such as temperature, pH, and salt concentration[10]. The NSA selects for an even smaller population of microorganisms than standard cultivation techniques detect[12, 14, 15], isolating only aerobic, spore-forming bacteria and not fastidious microbes and microbes that are currently uncultivable. Thus, in order to get a more comprehensive picture of the microbial burden in spacecraft assembly facilities and on flight hardware, culture-independent methods are needed to estimate and distinguish viable and dead microorganism populations.

Microscopy is one tool that is useful in distinguishing live and dead populations, however it is not a practical method for spacecraft and cleanroom surfaces due to the low abundance of microbes in these areas and high levels of debris, which promote false positive results from autofluorescence as well as issues with insufficient and nonspecific binding of strains[10, 16, 17]. Molecular methods, on the other hand, utilize universally common cellular compounds such as deoxyribonucleic acid (DNA) and adenosine triphosphate (ATP) for microbial detection and can be modified to distinguish the total and viable populations[9, 18-20]. Utilization of both molecular assays and traditional microbiology methods for future missions is ideal, but not practical due to the unavailability of large surface area of spacecraft surfaces required to collect enough biomass, and various project constraints such as mission schedule and budget. However, the spacecraft assembly facility (SAF) cleanroom environmental surfaces, where spacecraft are built, are available for analysis and can be used as a surrogate to conservatively estimate viable organisms that may be present on spacecraft.

Using the SAF, we can then determine a conservative ratio of NSA spores to viable microorganisms. This ratio can be used during the construction of the Mars 2020 mission to accurately estimate the number of viable microorganisms from the NSA samples that will be taken throughout the assembly, test, and launch operations (ATLO) activities. The Mars 2020 mission has requirements for limiting the probability of a viable organism from getting into a sample tube, to prevent false positive life detection when the samples are potentially returned to Earth via a currently unplanned future mission. Currently, a spore to VO ratio has been recommended based on assumptions from a 2006 Space Studies Board (SSB) report[14]. In this SSB report, it was estimated that the cultivable population represents 50x the spore population determined by NSA. It was also suggested that each microbial subpopulations' abundance is underestimated "by a factor of 1,000." Based on SSB recommendations, when cultivable organisms are viewed as a subset of the viable population, the ratio is assumed to be 1 spore to 50,000 VO[14]. This ratio was established with generalizations and approximations based on various aquatic and terrestrial environments, creating a need for an empirically-backed and standardized estimate for the Mars 2020 mission, based on samples taken in a

relevant environment such as the SAF. To accomplish this, three viability assays (internal ATP, PMA-qPCR, FACS) were used, along with the NSA, in this study to comprehensively estimate viable organisms and conservatively establish an empirically backed spore to VO ratio.

The ATP assay used in this study measures ATP, a key molecule found in living cells, using a commercially available ATP assay kit capable of measuring total and viable populations[21, 22]. This method has been validated for use on spacecraft and associated cleanroom surfaces[23, 24]. To distinguish between live and dead microbial populations, ATP eliminating agents, apyrase and adenosine deaminase, are used to eliminate ATP from dead and compromised cells leaving only ATP from viable cells to be measured[20]. Additionally, the ATP assay produces results in only 30 minutes for both total and viable populations. Currently, this assay is approved by NASA to preliminarily screen spacecraft surfaces, prior to spore assay sampling for enumeration of microbial contamination[23, 24]. ATP was used during the most recent rover mission, Mars Science Laboratory (MSL), prior to some of the nearly 5,000 NSA samples collected[25]. Additionally, the ATP assay has been routinely implemented to assess microbial burden of cleanroom floors associated with various missions[10, 11].

Another molecular method used in this study was quantitative polymerase chain reaction (qPCR), which specifically targets another biomolecule associated with life, DNA, to study the microbial population and is widely used in various kinds of environmental samples[26-28]. Quantitative polymerase chain reaction is a rapid molecular method that can be utilized by designing suitable gene marker(s) for the detection of specific species and broader populations of microorganisms. By modifying the NSA sample processing protocol, qPCR could be a valuable tool in differentiating TO and VO[26-28]. To distinguish between the live and dead microbial populations, DNA intercalating agents, such as propidium monoazide (PMA) are used. PMA interacts with naked DNA, and nucleic acids associated with dead and compromised cells, to prevent its amplification during PCR. The PMA-qPCR method, which has been successfully used in similar low biomass environments, can be utilized to measure microbial populations[26-28].

The third molecular assay used in this study was Fluorescent Assisted Cell Sorting (FACS), which is a flow cytometry method that can be used to measure VO[29]. Cells in a sample are stained with a suitable dye, such as RedoxSensor Green (RSG), which selectively stains viable cells that possess reductase activity. FACS can then look at a stream of individual cells and, with assistance of a fluorescent laser, can identify and enumerate VO labelled with RSG dye. Similar to the other molecular methods described above, FACS provides another option to estimate the viable population.

The main objective of this study was to utilize the molecule methods described above to calculate a spore to VO ratio, that will allow one to estimate the viable microbial burden using only NSA spore counts. Subsequent objectives were to provide a detailed understanding of the various populations in the Mars 2020 spacecraft assembly facility cleanroom (total, viable, cultivable, and spore) by spatial and temporal distribution. The conservative and empirically supported spore to VO estimation established in this study, in the low biomass environment of SAF, can then be applied to Mars 2020 spacecraft surfaces to help estimate viable organisms and meet the applicable planetary protection requirements.

II. Materials and Methods

A. Sample Location, Sampling and Processing

Over a period of six months, between March 2016 and August 2016, 98 floor samples were collected during 11 sampling time periods in the JPL SAF. The specific location for each sampling event and collection date are given in Figure 1. Total surface area of the SAF cleanroom is 921.1 m^2 with controlled conditions such as: temperature (20 ± 4°C), humidity (30 ± 5%), stringent gowning requirements, and weekly cleaning[26, 28]. Although SAF is capable of becoming an ISO-7 (10k) cleanroom, at the time of sampling SAF was certified as an ISO-8 (100k) cleanroom. A maximum measurement of 8,287 0.5 µm particles/ft^3 and 159 5.0 µm particles/ft^3 were seen during the 6 months of the study.

Each sample consisted of a 1 m^2 floor area in which particulates were collected using 9” × 9” polyester wipes (Texwipe; TX1009, NC, USA) and prepared as previously described[14]. Sampled wipes were deposited into sterile 500-mL glass bottles and transferred to an ISO-7 lab for further processing [30, 31]. Sterile phosphate buffer saline (PBS; pH 7.4; Sigma Aldrich, MO, USA) solution (200 mL) was added and thoroughly mixed for 30 seconds to release any collected particulates and associated microorganisms. The reaction mixture was concentrated to approximately 5 mL using an Innovaprep concentrating pipette with 0.45 µm Hollow Fiber Polysulfone (HFPS) concentrating pipette tips (Innovaprep Drexel, MO, USA). The exact amount of concentrated sample was weighed on a tared scale and appropriately recorded. Samples were then used for culture-dependent and culture-independent analyses as outlined below.

B. Culture-Dependent Microbial Examination

Several cultivation assays were employed to determine various microbial populations, as shown in Table 1. From the 5 mL of concentrated sample, an aliquot of 425 µl was subjected to heat shock treatment (80°C; 15 min) to estimate the spore abundance as per the NSA[31]. Suitable aliquots of non-heat shocked (NHS) samples were pour plated using tryptic soy agar (TSA; bacteria; 100 µl in quadruplicate) and potato dextrose agar (PDA; fungi; 100 µl in quadruplicate). The samples were then incubated at 32°C, and colony forming units (CFU) were counted after 24h, 48h, 72h, and 7 days of incubation time.

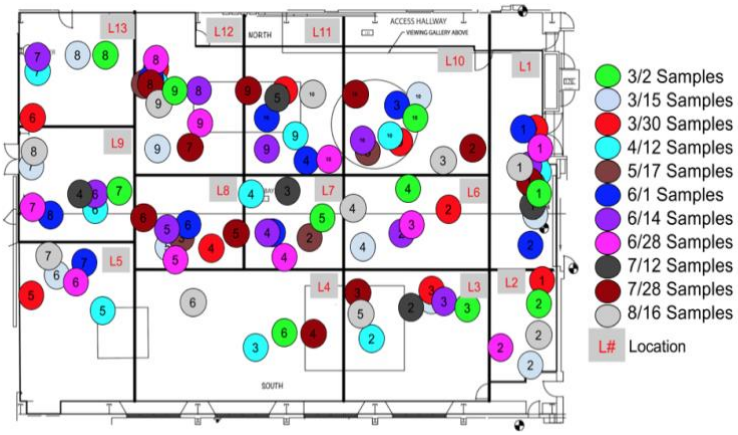

Figure 1. Schematic of the dates and locations sampled in the Spacecraft Assembly Facility. *A total of 98 samples were collected over a 6-month period from the SAF. The schematic above shows the date and the location of each sample that was collected. The circles represent the sample location and the number inside the circles represent the numerical order that the samples were taken. The color of the circle represents the corresponding day that the sample was collected. The graph is sectioned into artificial quadrants based on sample grouping and foot traffic, depicted by a grey box, to look for location specific differences in results. Dates are reported listed in the following format: Month/Date/Year.*

C. Culture-Independent Microbial Analyses

1. *ATP assay*

The total and intracellular ATP of samples was measured, as previously described, with the CheckLite HS ATP kit (Kikkoman, Japan)[30]. Total microorganisms (TO) were measured by lysing the cells with a detergent (benzalkonium chloride), followed by the measurement of photons using luciferin–luciferase enzyme reaction. In addition, the kit is capable of measuring internal ATP, a biomarker of VO, by eliminating free ATP and ATP associated with dead cells[9, 32]. This is accomplished via a brief apyrase/adenosine deaminase (ATP-eliminating) reagent step prior to the addition of benzalkonium chloride and measurement photon via the luciferin–luciferase reaction. The photon count, which is proportional to ATP concentration, was measured with a luminometer (Lumitester K-200, Kikkoman, Japan) as relative luminescence units (RLU).

2. *DNA Extraction*

A 3 mL aliquot was taken from the 5 mL concentrate of each sample and split into two, 1.5 mL portions. One aliquot was treated with 18.75 µl of 2 mM propidium monoazide (PMA) to a final concentration of 25 µM (2 mM; Biotium, Inc., Hayward, CA, USA) to perform the cell viability assessment[18, 28, 33, 34]. Each sample was vortexed and incubated in the dark for 5 minutes at room temperature. Samples were then exposed to PhAST Blue-Photo activation system for 15 minutes (GenIUL, S.L., Terrassa, Spain)[18, 28, 33, 34]. DNA was then extracted using a Maxwell 16 automated system (Promega, Madison, WI, USA), following manufacturer's instructions, and the resulting DNA suspensions (50 µl each) were stored at -20°C for further analysis[35].

3. *qPCR assay*

The qPCR assay was performed to estimate TO and VO by using the non-PMA and PMA treated samples, respectively. Samples were run in a CFX-96 thermal cycling qPCR instrument (Bio-Rad, California, USA). Universal bacterial primers targeting the 16S rRNA gene were 1369 F (5′-CGG TGA ATACGT TCY CGG-3′) and 1492 R (5′-GGW TAC CTTGTT ACG ACT T-3′)[31, 36]. Each 25 µl reaction in the 96 well plate consisted of 12.5 µl of 2 × iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 1 µl each of forward and reverse oligonucleotide primers (10 µM each), 9.5 µl DNase/RNase free water (Ultrapure, Gibco) and 1 µl of template DNA to be quantified. Reaction conditions were set to the following: 3 minute 95°C denaturation, followed by 39 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds[31].

4. *Fluorescent Assisted Cell Sorting*

Viable cell counting was performed as described elsewhere. Briefly, after initial sample collection, samples were preserved at -80°C in glyTE to preserve reductase activity until the samples could be processed. Once processing began, samples were diluted three-fold with filtered (0.2 µm pore size) 1x PBS and stained with RedoxSensor Green (RSG; Thermo Fisher Scientific) to identify VO. Individual particles that showed reductase activity were sorted using an inFlux sorter, with index sort capabilities, into three, 384-well plates, containing 0.6 µL of TE buffer per well. Negative control (64 wells) and positive control (3 wells) with 10 cells each were included in the plate. Cell diameters

were determined using the FACS light forward scatter signal, which was calibrated against cells of microscopy-characterized laboratory cultures[37]. Cell sorting and robotic liquid handling were performed in a cleanroom environment.

Table 1. Methods used to determine microbial burden from SAF, and the corresponding assumptions with each method.

Assay	Assumptions
Total ATP	• Detects ATP from both living and dead cells: fungi, bacteria, archaea, but not spores (minimal ATP in spores) • 1 RLU of ATP is equivalent to 1 CFU • Fungi = ~100 RLU/cell, Gram-positive = ~5 RLU/cell, Gram-negative = 1 RLU/cell, spores no ATP[9, 10] • Gram-positive and Gram-negative bacteria occur in equal proportion in SAF[9]
Internal ATP	• Detects only metabolically active, viable cells (VO): fungi, bacteria, archaea • ATP eliminating reagent enzymatically degrades free ATP • 1 RLU of ATP is equivalent to 1 viable CFU • Fungi = ~100 RLU/cell, Gram-positive = ~5 RLU/cell, Gram-negative = 1 RLU/cell, spores no ATP[9, 10]
16S rRNA qPCR	• Detects both living and dead cells (TO): only bacteria • 16S rRNA gene copy numbers per cell (1-15, average: 4.2, SD: 2.7)[38]
16S rRNA PMA-qPCR	• Detects living cells (VO): only bacteria • 16S rRNA gene copy numbers per cell (1-15, average: 4.2, SD: 2.7)[38] • Propidium monoazide (PMA) intercalates with free DNA and DNA from compromised cells preventing downstream amplification and detection
Non-Heat Shock	• Detects aerobic, cultivable bacteria that grow at 32°C on TSA • 1 CFU = 1 Cell
NSA Heat Shock	• Detects aerobic, cultivable spores that can survive 80°C for 15 minutes and grow at 32°C on TSA (NASA Standard Spore Assay) • 1 CFU = 1 Cell
FACS	• Detects viable cells capable of being stained with RSG+ dye and showing reductase activity (VO) • ~20 % of counts are positive for low coverage sequencing (LoCoS) • RSG+ dye is a conservative indicator of viability, has no known taxonomic bias, is compatible with cryopreserved sample analysis, has low background fluorescence, and is compatible with downstream genomics analysis.

D. Statistical Analysis

All statistical analyses were performed by Prism 7. Prism 7 was used to perform One-Way ANOVA (and Nonparametric) analysis with Tukey multiple comparisons. All statistical tests that had a $P=<0.05$ were considered significant.

III. Results

In total, 98 samples were collected over a period of six months from 13 different locations in the SAF and analyzed with cultivation, qPCR, ATP, and FACS based assessments.

A. Culture-Dependent Microbial Burden

The number of cultivable bacteria grown on TSA plates ranged from 1.2 x 10_1 to 6.6 x 10_3 CFU/m_2 and the number of cultivable fungi measured on PDA ranged from below detection limit (BDL) to 1.7 x 10_2 CFU/m_2. The average bacterial burden was 4.4 x 10_2 CFU/m_2 and fungal burden was 1.7 x 10_1 CFU/m_2. Similarly, the cultivable spore population, as per the NSA, ranged from BDL to 3.6 x 10_2 CFU/m_2 with an average of 3.6 x 10_1 CFU/m_2.

In order to assess the temporal and spatial distribution of the microbial population, samples were collected and analyzed on different days and at different locations around the cleanrooms (Figure 2). The highest cultivable burden observed for bacteria was on 6/1/16 (1.1 x 10_3 CFU/m_2), fungi on 3/1/16 (7.4 x 10_1 CFU/m_2), and spores on 5/17/16 (8.3 x 10_1 CFU/m_2). Similarly, the lowest observed burdens included bacteria on 3/1/16 (6.9 x 10_1 CFU/m_2), fungi on 6/14/16 through 8/15/16 (BDL), and spores on 6/28/16 (1.7 x 10_1 CFU/m_2). The largest microbial burden observed for the three cultivable populations measured in this study varied by location. The most abundant bacterial population was measured at location 1 (1.2 x 10_1 CFU/m_2), the largest fungal population was observed at location 10 (4.2 x 10_1 CFU/m_2), and the largest spore burden were seen at location 12 (7.2 x 10_1 CFU/m_2). Correspondingly, the lowest observed bioburden via location was shared between bacteria and spores at location 6 (1.8 x 10_2 CFU/m_2 and 1.8 x 10_1 CFU/m_2, respectively). The lowest spatial fungal burden was observed at location 11 (5.0 x 10_0 CFU/m_2). Aside from the statistically significant differences observed between fungi on 3/1/16 and eight other dates ($p<0.05$), no other statistically significant temporal or spatial distribution was noticed amongst the cultivable microbial populations.

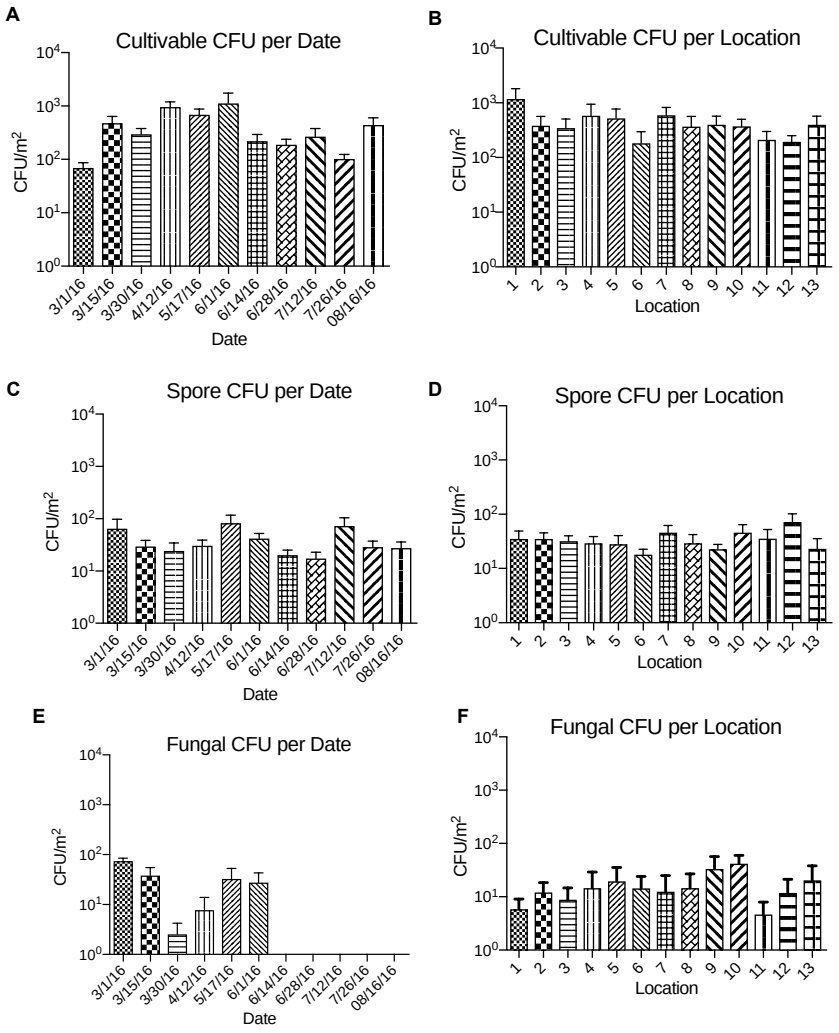

Figure 2. **Cultivable microbial burden at each date and location sampled in the SAF.** *Cultivable burden measured after 7 days of incubation as based on date: (A) bacteria (C) spores (E) fungi. Each bar represents the average of all samples collected at each sampling date. Cultivable burden measured after 7 days of incubation as based on location: (B) bacteria (D) spores (F) fungi. Each bar represents the average of all samples collected at each location (i.e. 1-13). Error bars for all graphs represent the standard error of the mean.*

B. Culture-Independent Microbial Burden and Diversity Analysis

1. ATP Assay

Of the 98 samples analyzed, 11 were below control values and were therefore not included in downstream analyses. The total ATP ranged from BDL to 4.2 x 10_6 RLU/m_2, with an average of 3.3 x 10_5 RLU/m_2 (data not shown) . The highest total ATP was seen on samples collected on 3/1/16 (1.4 x 10_6 RLU/m_2), which was significantly different than three other collection dates, including the date with the lowest average measurement (6/14/16; 5.1 x 10_4 RLU/m_2). When samples were compared spatially, the only significant difference ($p<0.05$) observed was between samples from

the highest average, observed at location 9 (1.4 x 10^6/m^2 RLU/m^2) and six other locations (1, 6, 8, 10, 11, 12). The lowest total ATP average was seen in location 4 (4.1 x 10^4/m^2).

The intracellular ATP from SAF floors averaged 2.7 x 10^4 RLU/m^2, which was equivalent to 5.6 x 10^3 – 2.8 x 10^4 viable cells/m^2. The temporal averages are shown in Table 2 and the spatial averages is shown in Table 3. The values ranged from BDL to 7.2 x 10^5 RLU/m^2. The only significant difference ($p<0.05$) observed by date was from samples collected on 3/30/16, which had the highest average of 1.3 x 10^5 RLU/m^2, and four other dates (4/12/16, 6/1/16, 6/14/16, 7/26/16). The lowest intracellular ATP contents were from 7/26/16 samples (1.5 x 10^3 RLU/m^2). There were no significant differences observed by location. The highest average of intracellular ATP was 8.2 x 10^4 RLU/m^2 for location 12 and the lowest was in location 4 (4.34 x 10^3 RLU/m^2). (Figure 3)

2. Quantitative PCR

Measured 16S rRNA gene copy numbers were recorded and additionally converted to CFU based on the average 16S rRNA gene copies per cell, 4.2 +/- a standard deviation of 2.7, for a range of 1.5 to 6.9 copies/cell[10]. Since >98% of the microbial burden was due to the bacterial abundance, the TO (data not shown) and VO values expressed below were for bacteria.

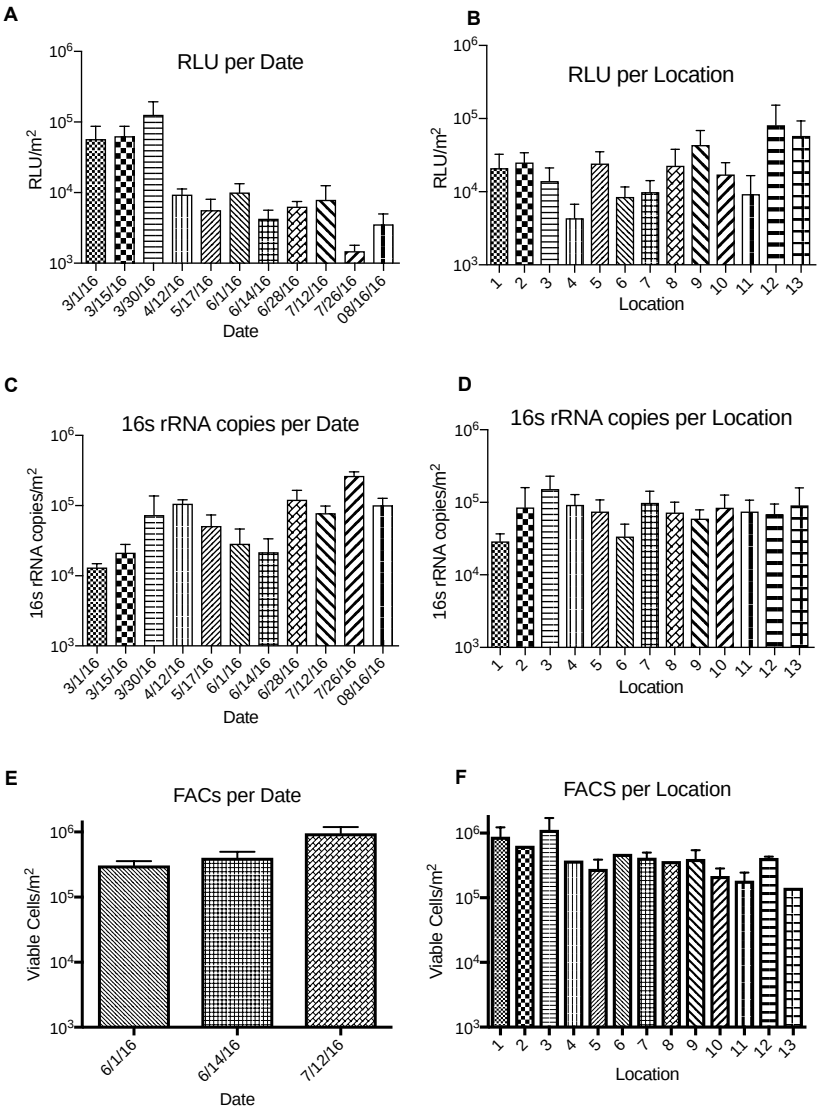

Figure 3. **Viable microbial burden at each date and location sampled in the SAF.** *Samples were analyzed via Internal ATP (A and B), PMA-qPCR (C and D) and FACS (E and F). Viable burden by date: (A) Intracellular ATP (C) PMA-qPCR (E) FACS. Each bar represents the average of all samples collected at each sampling date. Viable burden by location: (B) Intracellular ATP (D) PMA-qPCR (F) FACS. Each bar represents the average of all samples collected at each location (i.e. 1-13). Error bars for all graphs represent the standard error of the mean.*

The TO averaged 5.3 x 10^6 copies/m^2. This was equivalent to an estimated range of 7.7 x 10^5 – 3.6 x 10^6 cells/m^2. The range of copies/m^2 varied from 1.1 x 10^4 - 9.7 x 10^7 copies/m^2. A significant difference in TO was noticed between samples taken on 8/15/16 (2.0 x 10^7 copies/m^2) and seven other dates (3/1/16, 3/15/16, 3/30/16, 4/12/16, 6/1/16, 6/14/16, 7/26/16). The lowest TO average, 2.8 x 10^5 copies/m^2, was seen from 3/1/16 samples. When compared spatially, the average TO burden had no significant differences. The lowest TO average was seen from location 13 (1.7 x 10^6 copies/m^2) and the highest was observed in location 9 (1.6 x 10^7 copies/m^2).

Portions of the same samples that measured TO were analyzed after treatment with PMA dye to measure VO (Figure 3). The average VO was 7.9 x 10^4 copies/m^2 which was converted to an estimated range of 1.2 x 10^4 – 5.4 x 10^4 viable bacterial cells/m^2. The VO average was approximately 2-logs less than the TO average. The temporal distribution of VO is shown in Table 2 and the spatial distribution is shown in Table 3. The average VO varied slightly

between dates collected, but samples collected on 7/26/16 were significantly different ($p<0.05$) than all other dates. The lowest VOs were seen on 3/1/16 (1.3×10^4 cells/m^2) and the highest VOs on 7/26/16 (2.7×10^5 cells/m^2). Spatially, the lowest VO average was observed in location 1 (2.9×10^4 cells/m^2) and the highest VO average was seen in location 3 (1.5×10^5 cells/m^2).

Table 2. Microbial burden of Spacecraft Assembly Facility floors by date.

Sampling Date	Viable microbes‡‡ (RLU/m2) [B1]	Viable microbes‡‡ (16s/m2) [B2]	Cultivable‡ ‡ (CFU/m2) [C]	Cultivable Spores‡‡ (CFU/m2) [D]	Cultivable ATP %§§ [C/B1] x 100	Cultivable qPCR %§§ [C/B2] x 100	Spore %§§ [D/C] x 100	Spore to VO (ATP)⁎⁎⁎†† [B1/D]	Spore to VO (qPCR)⁎ ⁎⁎‡‡‡ [B2/D]
3/1/16	5.7×10^4	1.3×10^4	6.9×10^1	6.4×10^1	0.7	0.5	55.8	708	206
3/15/16	6.3×10^4	2.1×10^4	4.8×10^2	3.0×10^1	1.3	4.9	22.9	1,923	718
3/30/16	1.3×10^5	7.4×10^4	3.0×10^2	2.4×10^1	0.6	2.4	17.1	5,261	3,036
4/12/16	9.4×10^3	1.1×10^5	9.6×10^2	3.1×10^1	11.4	1.1	6.9	306	3,484
5/17/16	5.7×10^3	5.1×10^4	6.9×10^2	8.3×10^1	14.5	2.3	15.0	68	618
6/1/16	1.0×10^4	2.9×10^4	1.1×10^3	4.2×10^1	11.7	8.6	18.2	241	683
6/14/16	4.3×10^3	2.2×10^4	2.2×10^2	2.0×10^1	7.2	1.7	21.5	211	1,071
6/28/16	6.3×10^3	1.2×10^5	1.9×10^2	1.7×10^1	3.5	0.3	14.6	326	7,043
7/12/16	8.0×10^3	7.8×10^4	2.7×10^2	7.2×10^1	7.2	0.7	40.6	110	1,080
7/26/16	1.5×10^3	2.7×10^5	1.0×10^2	2.9×10^1	9.2	0.04	35.5	52	9,217
8/15/16	3.6×10^3	1.0×10^5	4.4×10^2	2.8×10^1	19.4	0.5	6.2	158	3,866
Average§§§	2.8×10^4	5.3×10^6	4.4×10^2	3.6×10^1	7.7	2.2	22.9	746	2,176

Table 3. Microbial burden of Spacecraft Assembly Facility floors by location.

Sample Location	Viable microbes* (RLU/m2) [B1]	Viable microbes‡‡ (Copies/m2) [B2]	Cultivable‡ ‡ (CFU/m2) [C]	Cultivable Spores‡‡ (CFU/m2) [D]	Cultivable ATP %† [C/B1] x 100	Cultivable qPCR %§§ [C/B2] x 100	Spore %§§ [D/C] x 100	Spore to VO (ATP)‡§ [B1/D]	Spore to VO (qPCR)⁎ ⁎⁎⁎⁎ [B2/D]
1	2.1×10^4	2.9×10^4	1.2×10^3	3.5×10^1	14.8	6.4	11.1	491	825
2	2.5×10^4	8.5×10^4	3.8×10^2	3.5×10^1	2.4	4.1	33.0	714	2,462
3	1.4×10^4	1.5×10^5	3.5×10^2	3.2×10^1	2.6	1.0	27.5	552	4,761
4	4.3×10^3	9.3×10^4	5.8×10^2	2.9×10^1	14.6	0.9	13.3	148	3,150
5	2.4×10^4	7.5×10^4	5.2×10^2	2.8×10^1	3.0	2.5	5.9	765	2,667
6	8.5×10^3	3.4×10^4	1.8×10^2	1.8×10^1	1.4	1.9	21.5	663	1,863
7	9.9×10^3	9.9×10^4	5.9×10^2	4.6×10^1	13.2	2.2	25.3	188	2,142
8	2.3×10^4	7.2×10^4	3.7×10^2	2.9×10^1	7.0	1.2	13.9	778	2,468
9	4.4×10^4	6.0×10^4	4.0×10^2	2.3×10^1	4.1	0.7	15.5	1,931	2,665
10	1.7×10^4	8.5×10^4	3.7×10^2	4.6×10^1	9.7	2.5	21.0	376	1,369
11	9.3×10^3	7.5×10^4	2.1×10^2	3.5×10^1	10.1	1.2	33.7	263	784
12	8.2×10^4	6.9×10^4	2.0×10^2	7.2×10^1	6.0	1.0	46.1	1,053	961
13	5.8×10^4	9.1×10^4	4.0×10^2	2.3×10^1	5.0	3.1	17.6	2,504	3,947
Average††	2.8×10^4	5.3×10^6	4.4×10^2	3.6×10^1	7.7	2.2	22.9	746	2,176

3. Fluorescent Assisted Cell Sorting (FACS)

Among 98 samples collected, only 25 were analyzed via FACS to estimate the number of viable microorganisms. Viable cells, as identified by FACS, were randomly sorted and 384 individual droplets were collected from each

⁎ Values calculated by taking the average of given assay values on individual sampling dates. See Materials and Methods for detailed explanation of individual assays.
† Percentage calculated using average of all samples calculated percentage values on a given sampling date. Individual samples that were BDL, were not included in the calculations.
‡ Ratio calculated for a given date by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a date had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.
§ Average represents a 1 RLU = 1 CFU assumption
⁎⁎ Average represents a 1 16S rRNA copy = 1 CFU assumption
†† Values are the average of all individual samples from the given dates. Spore to VO ratios were calculated by taking the sum of all samples from viable assays was divided by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

sample tested. Sequencing was performed on a single sample (Sample 1 from 7/12/16, location 1) to authenticate presence of the biological particles that were identified as viable during FACS. Out of the total number of viable cells that were sorted, only 20% were able to be sequenced and taxonomically assigned. However, for the most conservative approach, only the raw viable counts observed by FACS were used to generate the spore to VO ratio. A summary of the FACS-based VO population is summarized in Tables 4 and 5.

Table 4. Temporal distribution of FACS-based VO of SAF floors.

Sampling Location	Viable microbes (RSG+ CFU/m2)**** [B3]	Cultivable Spores (CFU/m2)**** [D]	Spore to VO (FACS)†††††††† [B3/D]	20 %Viable microbes (RSG+ CFU/m2)**** [B3]	20% Spore to VO (FACS)†††† [B3/D]
1	8.8 x 10^5	3.5 x 10^1	12,090	1.8 x 10^5	12,091
2	6.4 x 10^5	3.5 x 10^1	8,483	1.3 x 10^5	1,697
3	1.1 x 10^6	3.2 x 10^1	34,754	2.3 x 10^5	6,951
4	3.8 x 10^5	2.9 x 10^1	29,124	7.5 x 10^4	5,825
5	2.8 x 10^5	2.8 x 10^1	44,400	5.6 x 10^4	8,880
6	4.8 x 10^5	1.8 x 10^1	9,825	9.6 x 10^4	1,965
7	4.2 x 10^5	4.6 x 10^1	6,452	8.3 x 10^4	1,290
8	3.7 x 10^5	2.9 x 10^1	9,825	7.4 x 10^4	1,965
9	4.0 x 10^5	2.3 x 10^1	18,805	7.9 x 10^4	3,761
10	2.2 x 10^5	4.6 x 10^1	5,695	4.4 x 10^4	1,139
11	1.8 x 10^5	3.5 x 10^1	5,617	3.7 x 10^4	1,123
12	4.1 x 10^5	7.2 x 10^1	13,133	8.3 x 10^4	2,627
13	1.4 x 10^5	2.3 x 10^1	11,354	2.9 x 10^4	2,271
Average‡‡‡‡	4.8 x 10^5	3.6 x 10^1	12,091	9.5 x 10^4	12,091

Table 5. Spatial distribution of FACS-based VO of SAF floors.

Sampling Date	Viable microbes (RSG+ CFU/m2)* [B3]	Cultivable Spores (CFU/m2)**** [D]	Spore to VO (FACS)† [B3/D]	20% Viable microbes (RSG+ CFU/m2)**** [B3]	20% Spore to VO (FACS)†††† [B3/D]
6/1/16	3.1 x 10^5	4.2 x 10^1	7,338	6.1 x 10^4	1,468
6/14/16	4.0 x 10^5	2.0 x 10^1	19,889	8.1 x 10^4	3,978
7/12/16	9.6 x 10^5	7.2 x 10^1	13,218	1.9 x 10^5	2,644
Average‡	4.8 x 10^5	3.6 x 10^1	12,091	9.5 x 10^4	12,091

The average VO estimate from FACS analysis was 4.8 x 10^5 cells/m2. However, if a 20% factor is applied because only 20% of sorted "viable" particles were able to be amplified from sequence analyses, then the average estimate would be 9.5 x 10^4 viable cells/m2. Temporally, the VO population from FACS analysis varied from the lowest value on 6/1/16 (3.1 x 10^5/m2) to the highest value, seen on 7/12/16 (9.6 x 10^5 cells/m2). The 7/12/16 values were significantly different ($p<0.05$) than 6/1/16 and 6/14/16 samples. When spatial distribution of the VO population was analyzed, the lowest value was at location 13 (1.4 x 10^5 cells/m2) and the highest VO was measured at location 3 (1.1 x 10^6 cells/m2).

C. Spore to VO ratios

A spore to VO ratio was calculated for the three viability assays employed during this study: ATP, qPCR, and FACS. Each assay was compared with the NSA spore counts to calculate a spore to VO ratio (Figure 4).

* Values calculated by taking the average of given assay values on individual sampling locations. See Materials and Methods for detailed explanation of individual assays.
† Ratio calculated for a given location by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation
‡ Values are the average of all individual samples from the given dates. Spove to VO ratios were calculated by taking the sum of all samples from viable assays was divided by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

The ATP based spore to VO ratio was established by taking the sum of all internal ATP RLU/m2 values and dividing by the sum of all spore CFU/m2 values. A range was then created from the average value by separately dividing the average by 1 and 5 to account for the different RLU/cell for gram-negative and gram-positive positive. Spore values from samples that had below control internal ATP measurements were not included in this calculation. The spore to VO measured by intracellular ATP range was 149 to 746. No significant differences were seen spatially. Location 13 showed the highest spore to VO range (501 to 2,504) and location 4 showed the lowest spore to VO range (30 to 148). The spore to VO ratio measured by ATP assay showed significant ($p<0.05$) temporal distribution when samples were compared (3/15/16 with 4/12/16, 5/17/16, 6/1/16, 6/14/16, 6/28/16; 3/30/16 with 6/1/16, 6/14/16; 3/30/16, 7/26/16). The highest range was observed on 3/30/16 (1,052 to 5,261) and the lowest ratio range documented on 7/26/16 (10 and 52).

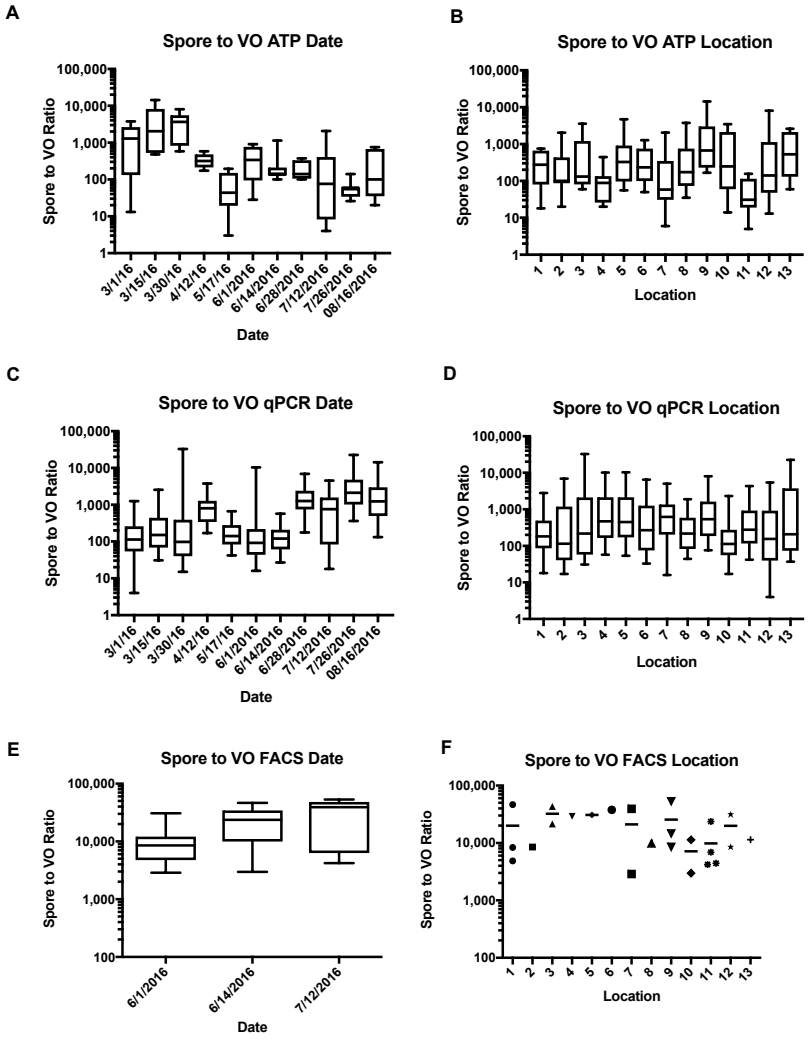

Figure 4. Spore to viable organism ratio as determined by Internal ATP, bacterial 16S rRNA gene PMA-qPCR, and FACS by date and location in SAF. *The spore to VO ratio was calculated by dividing the sum of assay specific values/m2 by the sum of spore/m2 of the same samples. For ATP spore to VO ratios, two numbers were included for each sample to account for the equal abundance of gram positive (5 RLU = 1 CFU) and gram negative cells (1 RLU = 1 CFU)[9,10]. This ATP spore to VO range was calculated by sampling date (A) and sampling location (B). For PMA-qPCR based spore to VO ratio, 3 numbers were included for each individual sample. They are minimum, maximum, and the average 16S rRNA copies per cell (4.2 +/-; standard deviation of 2.7)[38]. The PMA-qPCR based spore to VO ratio is shown by date (C) and location (D). The FACS based spore to VO ratio is shown by date (E) and location (F). Each box plot shows the minimum and maximum values (whiskers) and first quartile, third quartile, and median (box).*

The qPCR based spore to VO ratio was established by taking the sum of all PMA-qPCR 16S rRNA gene copies/m2 values and dividing by the sum of all spore CFU/m2 values. The calculated spore to VO value of 2,176 was then converted to a range by accounting the numbers of 16S rRNA gene per bacterial cell (4.2 ± one standard deviation, 2.7). The resulted ratio spore to VO ratio was in the range of 314 to 1,491. No significant differences were observed in this ratio by samples collected either date-wise or location-wise. The highest range was on 7/26/16 (1,332 to 6,339) and the lowest on 3/1/16 (30 to 141). Location 3 showed the highest spore to VO range (688 to 3,274) and the lowest for location 11 (113 to 539).

The FACS based spore to VO ratio was determined by using the sum of FACS-based viable counts and dividing by the sum of the applicable spore CFU/m^2 values. When the raw FACs viable counts are used, the ratio is 12,091. Of the three dates observed, there were significant differences ($p<0.05$) between 7/12/16 and samples collected from 6/1/16 and 6/14/16. There were no significant differences by location.

IV. Discussion

The standard method to assess microbial burden on spacecraft and its associated environmental surfaces is designed to measure cultivable spores using the NSA method. The spore counts measured during this study were consistent with those obtained from previous studies[10, 20] (Table 6). Notably, average spore counts were observed across various locations in the SAF within the same order of magnitude (~10^1 CFU/m^2). However, when compared between individual samples, differences in spore counts were in the range of 2% to 99% variation from the average. This would suggest that spore populations fluctuate nominally at various locations spatially, and further confirmed that there are temporal variations potentially related to assembly activities and human traffic. The routine cleaning and maintenance procedures followed in JPL-SAF are effective at reducing the spore burden but not in eradicating them. In contrast, the average cultivable bacteria burden showed variability of up to two logs between each sample collection date. Spatially, the cultivable populations were much more consistent. However, location 1, the site that sees the most foot traffic, had an average cultivable bioburden approximately double that of any other individual location. Moreover, cultivable fungi were detected in similar low quantities as that of spores between sampling dates 3/1/16 and 6/1/16, but were not recovered from 6/14/16 onward. Spatially, fungi were consistently distributed in low quantities in all 13 locations.

In addition to the cultivation based assays, a variety of molecular methods were utilized to assess microbial burden. Compared to other non-systematic studies of SAF, ATP and qPCR values obtained in our study had a slightly larger range of VO[10, 26, 28, 39] (Table 6). This could be the result of our larger sample size, which would potentially increase the likelihood of finding a microbial rich sampling location that was not detected by the prior studies.

Throughout this study, we observed that the internal ATP based VO estimates were consistently lower than PMA-qPCR based VO estimates both spatially and temporally. This could potentially be explained by the inherent lower metabolic activity demonstrated by microbes in the cleanroom environment, along with the reduced metabolic activity caused by the floor cleaning reagents used during weekly cleaning in SAF. Although ATP would be effected in these conditions, VO expressing lower metabolic activity would still possess the gene copies that are detected by the qPCR methods. Furthermore, it has been previously suggested that the shorter half-life of ATP, compared with DNA, could also be responsible for lower ATP values[26].

This was the first time that the third molecular assay, FACS, was used in a low biomass environment to assess viable burden. Although the floor samples of SAF were low in biomass, FACS was able to generate VO estimates. When these samples were further analyzed and sequenced (data not shown), only 20% of the cells were shown to be amplifiable and capable of being assigned taxonomic affiliations. Since the whole genome amplification was performed in order to generate enough material to sequence for the taxonomic identity, other factors, such as too low of a DNA concentration or DNA extraction procedures that are not compatible with cells from hardy populations, might explain the FACS results. However, FACS studies previously conducted by others exhibited similar percentage outcomes as was observed during this study[29, 40-42].

Although spacecraft cleanroom microbial communities have been shown to be dominated by bacteria, it is important to include estimates of the fungal and archaea populations[43]. The fungal population was estimated in this study by fungal qPCR (data not shown) and cultivation on Potato Dextrose Agar (PDA). These estimations showed that the fungal populations in SAF were ~ 2% of the microbial community which were consistent with other studies[44, 45]. Archaeal populations, although not analyzed directly in this study, have previously been shown to exist in extremely low quantities (BDL-1%) in spacecraft cleanrooms[44]. Although the combined fungal and archaeal communities have a small presence in SAF, it is important to understand the entire viable microbial population in order to assess contamination for spacecraft.

Table 6. Microbial burden of Spacecraft Assembly Facility surfaces.

Assay (Units)	This Study	Previous Studies
Cultivable (CFU / m^2)	1.2×10^1 – 6.6×10^3	BDL– 5.4×10^5 [10, 20]
Spores (CFU / m^2)	BDL – 3.6×10^2	BDL– 4.0×10^3 [10, 20]
Total ATP (RLU / m^2)	BDL – 4.2×10^6	BDL – 4.5×10^6 [10, 20, 26]
Internal ATP (RLU / m^2)	BDL – 7.2×10^5	1.4×10^3– 4.8×10^4 [10, 26]
Total qPCR (copies / m^2)	1.1×10^4 – 9.7×10^7	BDL – 3.3×10^7 [10, 26, 28]

PMA qPCR (copies / m^2)	$3.8 \times 10^3 – 6.5 \times 10^5$	$6.2 \times 10^3 – 4.9 \times 10^4$ [26, 28]
FACS (CFU / m^2)	$8.7 \times 10^4 – 1.7 \times 10^6$	-

V. Conclusion

Three viability assays were used to establish a ratio between spore counts and VO to compare with the SSB estimation (1 spore to 50,000 VO)[14]. To get the most conservative and comprehensive assessment, three different methods widely used in the field of microbial ecology were utilized in this study. As demonstrated in Section III, internal ATP and PMA-qPCR provided the lowest spore to VO estimates with the ATP based estimate of 1 spore to 149 – 746 VO and the qPCR based estimation of 1 spore being equal to 314 – 1,491 VO. The third viability assay used in this study, FACS, provided the most conservative spore to VO estimation of 1 spore being equivalent to 12,091 VO. Based on the empirical data generated during this study and the desire to utilize the most conservative method, it is recommended that the FACS based ratio of 1 spore being equal to 12,091 VO be used as a replacement to the SSB estimate for samples collected from Mars 2020 and associated SAF surfaces. This ratio provides an estimate supported by data collected in a relative environment and should be used to replace the SSB estimate that was created based off of generalizations and assumptions observed in various environments.

It is also important to consider that this conservative ratio is representative of SAF floors at JPL and could potentially be different in other spacecraft cleanrooms, and on spacecraft surfaces. In fact, microbial burden on cleanroom floors has previously been shown to be typically two orders of magnitude greater than spacecraft surfaces[17]. This could potentially be answered with additional studies in those environments. In addition to the results presented here, planned future analysis will investigate iTag-based microbiome and multigene-based metagenomics study to further explore the microbial burden and diversity of the SAF environment.

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